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Effect of Pyruvate Carboxylase Overexpression on the Physiology of *Corynebacterium glutamicum*

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Pyruvate carboxylase was recently sequenced in *Corynebacterium glutamicum* and shown to play an important role of anaplerosis in the central carbon metabolism and amino acid synthesis of these bacteria. In this study we investigate the effect of the overexpression of the gene for pyruvate carboxylase (*pyc*) on the physiology of *C. glutamicum* ATCC 21253 and ATCC 21799 grown on defined media with two different carbon sources, glucose and lactate. In general, the physiological effects of *pyc* overexpression in *Corynebacteria* depend on the genetic background of the particular strain studied and are determined to a large extent by the interplay between pyruvate carboxylase and aspartate kinase activities. If the pyruvate carboxylase activity is not properly matched by the aspartate kinase activity, *pyc* overexpression results in growth enhancement instead of greater lysine production, despite its central role in anaplerosis and aspartic acid biosynthesis. Aspartate kinase regulation by lysine and threonine, pyruvate carboxylase inhibition by aspartate (shown in this study using permeabilized cells), as well as well-established activation of pyruvate carboxylase by lactate and acetyl coenzyme A are the key factors in determining the effect of *pyc* overexpression on *Corynebacteria* physiology.

Corynebacteria belong to the large group of gram-positive bacteria with a high G+C content, which constitutes the *Actinomyces* subdivision together with genera such as *Streptomyces, Propionibacteria*, and *Arthrobacter.* Certain saprophytic corynebacteria, such as *Corynebacterium glutamicum* and its close relative *Brevibacterium flavum* are used predominantly in industrial fermentation processes, such as lysine production, because of the almost complete lack of regulation of their lysine biosynthesis metabolic pathway and their high capability for lysine excretion (7, 8). Lysine is used in increasing volumes as a food additive for poultry and pig breeding (2), a clear reflection of the improvement of living conditions around the world.

Extensive studies during the past 15 years have enhanced our understanding of metabolic flux distribution and control in *C. glutamicum* during lysine fermentations. These studies led to the identification of phosphoenolpyruvate/pyruvate as a critical branch point, controlling the supply of anaplerotic carbon for the biosynthesis of aspartic acid family amino acids. Oxaloacetate replenishment in particular was determined to be a critical step in lysine production (38, 42, 43). The first anaplerotic enzyme to be investigated in this context was phosphoenolpyruvate carboxylase (PEPC), whose presence has been well established in *C. glutamicum* previously (9, 10, 27). Using a PEPC-deficient mutant strain (*ppc*$^-$), it was shown that PEPC is dispensable in lysine production and has little effect on cell growth (11, 30). Furthermore, a double mutant (*ppc*$^-$ *pyk*$^-$) showed a substantial reduction of both growth and lysine productivity (28, 29), pointing to the importance of pyruvate in supplying anaplerotic carbon for growth and lysine production. These results suggested that alternative anaplerotic pathways, which most probably use pyruvate as a substrate, operate in corynebacteria. Further evidence for the direct carboxylation of pyruvate was provided by the development of an independent enzymatic assay that utilized permeabilized cells (31, 41). The presence of pyruvate carboxylase was finally confirmed by sequencing the *pyc* gene in corynebacteria (18, 32). Studies with the *pyc* deletion mutant as well as the *pyc ppc* double mutant showed that pyruvate carboxylase is the essential anaplerotic pathway and that no further anaplerotic pathways exist in *C. glutamicum* (32). However, despite the important role of this enzyme in growth and product formation, no results on its physiological effects have been reported.

In the present study, we have investigated the effect of *pyc* overexpression on *C. glutamicum* physiology, especially in terms of growth and lysine production. Overexpression of pyruvate carboxylase must be studied in conjunction with other enzymes synthesizing or depleting metabolites that regulate their respective activities. Of particular importance is aspartate kinase and, by extension, the genetic background of the strain determining the regulation of these enzymes. We report here our findings with *pyc* overexpression in two different strains: *C. glutamicum* ATCC 21253, which has a regulated aspartate kinase, and *C. glutamicum* ATCC 21799, which has a deregulated aspartate kinase (15), and for two different carbon sources, glucose and lactate.

MATERIALS AND METHODS

Strains and media. Two strains, *C. glutamicum* ATCC 21253, auxotrophic for L-homoserine (or L-threonine plus L-methionine) and L-leucine, and *C. glutamicum* ATCC 21799, auxotrophic for L-leucine and pantothenate and aminoethylcysteine resistant (AEC^r), were used in this study. For convenience, *C. glutamicum* ATCC 21253 and ATCC 21799 are referred to simply as 21253 and 21799, respectively. Other strains are summarized in Table 1. The defined medium developed by Kiss and Stephanopoulos (17) was used as a basal medium supplemented with 10 mg of pantothenate per lites for 21799. For some experiments focusing on examining the effect of the carbon source, 20 g of lactate per liter was used as a carbon source instead of glucose.

Analysis of cell mass, carbon sources, and intra- and extracellular amino

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TABLE 1. Strains used in this study

Strain	Genotype and characteristics	Reference or source
C. glutamicum		
21253	*hom*	26
21253(pKD7)	*hom pyc::kan*	This study
21799	AEC^r	19
21799(pKD7)	AEC^r, *pyc::kan*	This study
E. coli		
DH5α	*hsdR recA*	12

acids. Cell mass was measured by using a UVIKON 930 spectrophotometer (Research Instruments, San Diego, Calif.). Dry cell weight was estimated based on the correlation 1 OD_{600} unit = 0.28 g dry cell weight (DCW)/liter. The amount of L-lysine accumulated in the medium and the amount of remaining L-threonine were quantitatively measured as *o*-phtalaldehyde derivatives (16) on a Hewlett-Packard (Waldronn, Germany) 1050 high-performance liquid chromotography HPLC system equipped with an AminoQuant column (Agilent Technologies, Wilmington, Del.). The levels of intracellular amino acids were measured in the extracts of the cells inactivated by silicon oil centrifugation to produce a cytosolic fraction of metabolites based on the method developed by Rottenberg (35) using silicon oil (Sigma, St. Louis, Mo.) and a glass bead beater (Biospec, Washington, N.C.). Glucose and lactate levels in the medium were measured by using the corresponding kits for each carbon source purchased from Boehringer (Mannheim, Germany).

Plasmid construction. Three different plasmids were constructed, allowing insertion of upstream and downstream untranslated regions of different sizes along with the structural *pyc* gene. The *Escherichia coli-C. glutamicum* vector pMAGK (−) was used for gene cloning in *C. glutamicum.* This vector was constructed based on the multiple-cloning site of the vector pMAL-p2X (New England Biolabs, Beverly, Mass.) and the broad-host-range replication site pEP2 (24).

For the first construct, cosmid IIIF10, which was used to obtain the *pyc* sequence (18), was digested with *Hin*dIII and the *pyc* gene was cloned as a 9-kb fragment into the *E. coli* vector pCR-Script as well as the *E. coli-C. glutamicum* vector pMAGK(−), giving rise to plasmids pCR9pc-Script and pMAGK9pc, respectively. Plasmid pMAGK9pc was then transformed into strain 21799. For the second construct, plasmid pCR9pc-Script was digested with 18 restriction enzymes that do not affect the *pyc* gene based on its restriction map: *Afl*II, *Apa*I, *Avr*II, *Bsp*HI, *Dra*I, *Eco*RV, *Hin*dIII, *Kas*I, *Nco*I, *Nde*I, *Nhe*I, *Nsp*I, *Sac*I, *Spe*I, *Sph*I, *Ssp*I, *Xba*I, and *Xmn*I. Using DNA electrophoresis, several bands were detected after the digest, one of which (a 4-kb DNA fragment) contained the *pyc* gene (identified by PCR). The 5′ and 3′ ends of this DNA fragment (possible sticky) were blunted using *Pfu* DNA polymerase as described by the manufacturer (Stratagene Inc., La Jolla, Calif.), and the fragment was then introduced into vector pMAGK(−), giving rise to plasmid pMAGK4pc. This plasmid was also introduced into strain 21799.

For the third construct, cosmid IIIG7, one of the four cosmids containing the *pyc* gene (18) was first digested with *Hin*dIII, yielding a 12-kb DNA fragment that contains the *pyc* gene. This fragment was introduced into vector pCR-Script, generating plasmid pCR12pc-Script. The latter was next digested with *Sca*I-*Ssp*I, and the resulting 7-kb DNA fragment containing the pyruvate carboxylase gene was introduced into vector pMAGK(−), generating plasmid pKD7. This plasmid was transformed into strains 21799 and 21253, generating strains 21799(pKD7) and 21253(pKD7), respectively. The resulting three constructs of plasmids are described briefly in Fig. 1.

DNA preparation and transformation. Restriction enzymes, T4 DNA ligase, and reagents were purchased from New England Biolabs or Boehringer Mannheim (Indianapolis, Ind.). Cosmid and plasmid DNA were prepared using Qiaprep spin columns (Qiagen, Valencia, Calif.), and DNA was extracted from agarose gels with the Qiaex kit (Qiagen). Plasmid DNA from *C. glutamicum* was isolated as described by Colon (6). *E. coli* was transformed by the $CaCl_2$ method (36), and *C. glutamicum* was transformed by electroporation as described by Liebl et al. (22).

Pyruvate carboxylase assay. The activity of pyruvate carboxylase was determined using permeabilized *C. glutamicum* cells by the method of Uy et al. (41). Cells collected from the culture, cultivated for 9 h (just before the depletion of threonine in the medium), were washed twice with 20 ml of 50 mM Tris-HCl buffer (pH 6.3) containing 50 mM NaCl, resuspended to an optical density at 600 nm (OD_{600}) of about 150 in 10 mM EDTA buffer (pH 7.4) containing 20% (vol/vol) glycerol, and then frozen at −20°C.

For the permeabilization step, the frozen cells were slowly thawed on ice and then mixed with a solution of 2.5% (wt/vol) hexadecyltrimethylammonium bromide (CTAB), resulting in a 0.3% (wt/vol) final CTAB concentration. The duration of permeabilization was about 1 min, which was previously reported to be the optimal permeablization duration (41).

The permeabilized-cell suspension was immediately used to assay pyruvate carboxylase activity from the measured transformation rate of pyruvate into oxaloacetate. A proper amount of each permeabilized-cell suspension was added to 1 ml of a reaction mixture containing 100 mM Tris-HCl (pH 7.3), 25 mM $NaHCO_3$, 5 mM $MgCl_2$, 3 mM pyruvate, and 4 mM ATP. To find the appropriate activity of each cell five different amounts of permeabilized cells, 0.5, 1, 2, 3, and 4 mg, were incubated for three different reaction periods, 0.5, 1, and 2 min, at

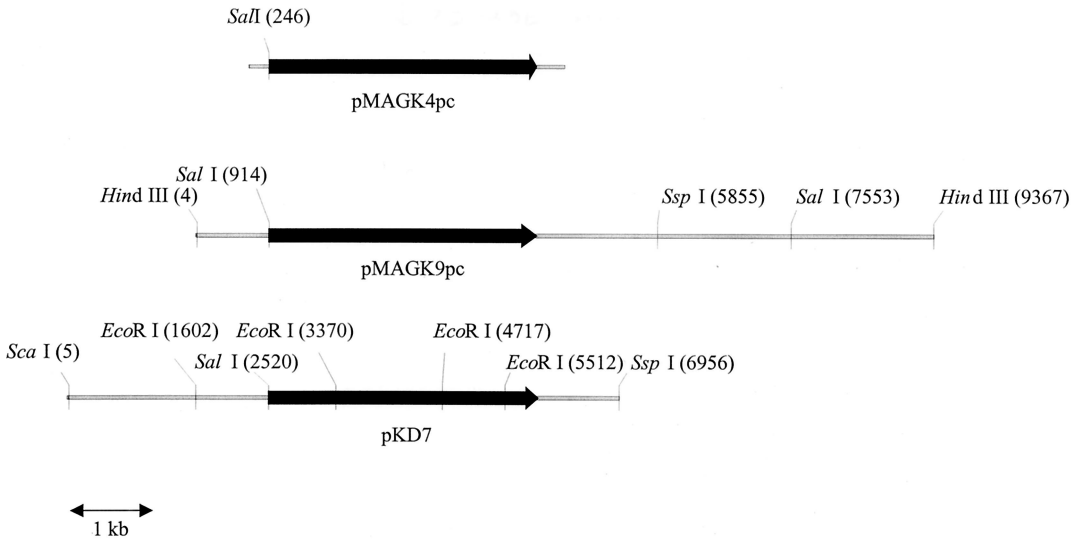

FIG. 1. Three constructs containing the pyruvate carboxylase gene and different sizes of untranslated regions used in this study. The *C. glutamicum-E. coli* plasmid pMAGK(−) was used as the vector for the constructs shown. Thick arrows describe the structural genes of pyruvate carboxylase, and thin lines represent the untranslated regions. Numbers in parentheses are the positions of the restriction sites from the beginning of each construct.

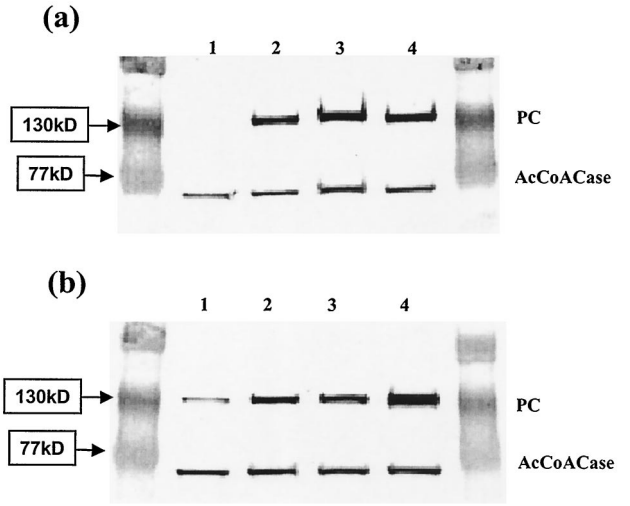

FIG. 2. Western blot of pyruvate carboxylase expressed in the parental and recombinant 21799 strains harboring three different plasmid constructs of *pyc* genes. Avidin-alkaline phosphatase was used for the detection of the biotinylated enzymes. (a) Cells grown on glucose; (b) cells grown on lactate. Lanes: 1, parental strain; 2, pMAGK4pc; 3, pMAGK9pc; 4, pKD7. AcCoACase, acetyl-CoA carboxylase.

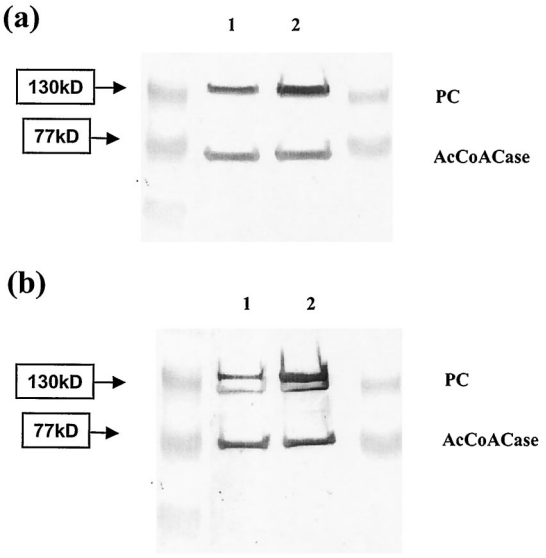

FIG. 3. Western blot of pyruvate carboxylase expressed in the parental and recombinant 21253 strains harboring pKD7 grown on glucose (a) or lactate (b). Lanes: 1, parental; 2, pKD7. AcCoACase, acetyl-CoA carboxylase.

30°C. The reaction was stopped by addition of 80 μl of 30% (wt/vol) *o*-phosphoric acid, and cell debris was removed by centrifugation (25,000 × *g* at 4°C for 15 min). In each sample, the unconverted pyruvate concentration was determined by a colorimetric method using a pyruvate assay kit purchased from Sigma Diagnostics Inc. One unit of pyruvate carboxylase activity was defined as the amount of enzyme converting 1 nmol of pyruvate per min.

Detection of in vivo-biotinylated proteins. Protein extracts from different strains (*C. glutamicum* and *E. coli*) were first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE (7% [wt/vol] acrylamide) and transferred to a nitrocellulose membrane. Biotinylated proteins were then detected with avidin-alkaline phosphatase conjugate (Bio-Rad, Hercules, Calif.) directly on the membrane as previously described (18). Two biotinylated enzymes were detected: one in the region of 70 kDa, which has previously been shown to correspond to the biotinylated subunit of acetyl coenzyme A (CoA) carboxylase (31, 32), and one in the region of 120 kDa, corresponding to pyruvate carboxylase (18).

Cell cultures. All bacterial cultures in this study were conducted with 50 ml of defined medium (described above) in 300-ml shake flasks incubated at 30°C with rotation at 220 rpm. To measure the cell mass and extracellular amino acid production, samples of 1 ml each were taken from the culture broth at the proper cultivation time during the incubation. For the measurement of pyruvate carboxylase activity and intracellular amino acid concentrations, cells were harvested 9 h after the start of the cultivation (just before the depletion of threonine in the culture broth) and measurements were made as described above.

RESULTS

Overexpression of pyruvate carboxylase in corynebacteria. Overexpression of pyruvate carboxylase in *C. glutamicum* was achieved using three different plasmid constructs, each containing the *pyc* gene in a different-size DNA fragment. Pyruvate carboxylase polypeptides were detected by Western blot analysis (Fig. 2). For cell cultivation on glucose (Fig 2a), all three recombinant strains showed similar amount of polypeptide synthesis. However, when lactate was used as the carbon source, different amounts of polypeptides were detected for the different plasmid constructs transformed into strain 21799 (Fig. 2b). The strain harboring plasmid pKD7 [21799(pKD7)], containing the *pyc* gene together with a 2.3-kb upstream DNA region, showed the largest amount of pyruvate carboxylase polypeptide. Due to the significant difference in *pyc* overexpression obtained with plasmid pKD7, strains harboring this plasmid were used in the subsequent pyruvate carboxylase overexpression studies.

Overexpression of the *pyc* gene was also obtained by transforming strain 21253, as shown in Fig. 3.

Pyruvate carboxylase activities in *pyc*-overexpressing corynebacteria. Table 2 summarizes the pyruvate carboxylase activities for the two recombinant strains cultivated on two carbon sources, glucose and lactate. It can be seen that the use of lactate invariably produces higher pyruvate carboxylase activities, which is in accordance with observations reported before (31, 41). We found that when the recombinant strains were grown on lactate, their pyruvate carboxylase activity was greater regardless of the type of host cell used. While the same behavior was observed with strain 21799 on glucose, no difference in pyruvate carboxylase activity was observed between the recombinant and parental strains in strain 21253 grown on glucose. A major difference between strains 21253 and 21799 was the feedback inhibition of aspartate kinase in strain 21253, as a result of which it is possible that aspartate accumulates

TABLE 2. Comparison of pyruvate carboxylase activities in *C. glutamicum* ATCC 21253 with those in C. glutamicum ATCC 21799 on different carbon sources

Strain	Pyruvate carboxylase activity (U/mg DCW ± SD) on:	
	Glucose	Lactate
21253	58 ± 5	97 ± 7
21253(pKD7)	58 ± 6	161 ± 10
21799	30 ± 4	36 ± 3
21799(pKD7)	53 ± 7	89 ± 8

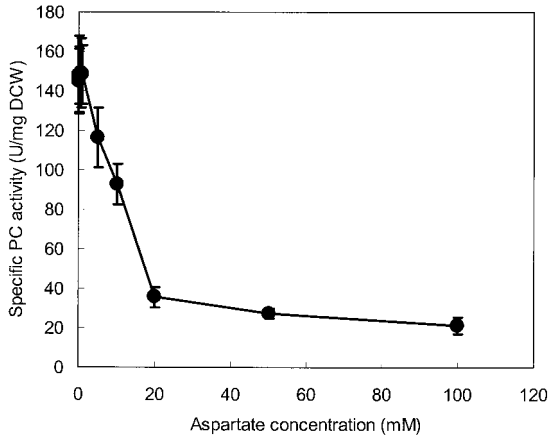

FIG. 4. Effect of aspartate on pyruvate carboxylase activity in permeabilized *C. glutamicum* cells.

intracellularly in this strain and feedback inhibits pyruvate carboxylase. Such would not be the case for strain 21799, where no negative regulation was exercised on aspartate kinase by threonine and accumulating lysine. Since aspartate is a common inhibitor of pyruvate carboxylases, the in vivo pyruvate carboxylase activity should be attenuated in strain 21253 compared to strain 21799. To investigate this hypothesis, the effect of aspartate on pyruvate carboxylase activity was also studied.

Aspartate inhibits pyruvate carboxylase activity in *C. glutamicum*. Since differences in aspartate kinase regulation in strains 21253(pKD7) and 21799(pKD7) could lead to different intracellular aspartate levels, the effect of aspartate on pyruvate carboxylase activity was investigated by using the permeabilized-cell activity assay. As shown in Fig. 4, aspartate strongly inhibited pyruvate carboxylase. The pyruvate carboxylase activity decreased sharply as the aspartate concentrations increased up to 20 mM, with a slower effect observed beyond this point.

Intracellular aspartate accumulation was next measured in various *C. glutamicum* strains (Table 3). For this purpose, strains 21253, 21799, 21253(pKD7), and 21799(pKD7) were grown on lactate or glucose minimal medium and cells were harvested at late exponential phase, just before threonine was depleted from the medium. A higher intracellular aspartate level was generally detected in strains 21253 and 21253(pKD7), which express the wild-type-regulated aspartate kinase enzyme, than in strains 21799 and 21799(pKD7), which express a deregulated aspartate kinase. Furthermore, the use of lactate as a carbon source resulted in generally higher intracellular aspartate levels than those obtained using glucose. These results support the hypothesis that aspartate kinase regulation by threonine and lysine in strain 21253 leads to intracellular aspartate accumulation that, in turn, inhibits the pyruvate carboxylation activity in host cells of strain 21253.

Effect of pyruvate carboxylase overexpression and carbon source on cell physiology. The previously established effect of carbon sources and the genetic background of the host cell on the pyruvate carboxylase activity was expected to affect macroscopic cell physiology as well. To investigate such effects, strains 21253, 21253(pKD7), 21799, and 21799(pKD7) were grown in either glucose or lactate minimal medium and their growth and lysine production profiles were examined.

TABLE 3. Intracellular aspartate concentration in different *Corynebacterium* species

Strain	Intracellular aspartate concn (μmol/g DCW ± SD) on[a]	
	Glucose	Lactate
21253	30.7 ± 4.70	65.8 ± 4.57
21253(pKD7)	48.4 ± 1.91	83.0 ± 4.56
21799	16.6 ± 4.84	22.8 ± 4.32
21799(pKD7)	18.3 ± 4.36	23.6 ± 2.65

[a] Intracellular aspartate concentrations were measured in cells harvested at 9 h.

When the strains were grown on glucose, no significant differences in cell growth rate or lysine production were observed between the parental 21253 strain and its recombinant derivatives, except for an extended lag phase in the profile of lysine accumulation (Fig. 5a). On the other hand, strain 21799(pKD7) grew faster than parental strain 21799 on glucose and the final cell concentration of the recombinant strain was twice that of the parental strain (Fig. 5b). The specific lysine production by the recombinant 21799(pKD7) strain was lower than that by the parental strain, even though the final lysine concentration in the fermentation broth was approximately the same (2 g/liter) for both strains (Fig. 5b).

When lactate was used as carbon source, both recombinant strains 21253(pKD7) and 21799(pKD7) grew faster but produced less lysine than the parental strains (Fig. 6). In terms of maximum growth rate and maximum specific lysine production rate, the effect of pyruvate carboxylase overexpression appeared to be similar for both strains when grown on lactate (Table 4). This trend was the same when the cells were grown on glucose, except for strain 21253, where *pyc* overexpression did not seem to have any effect on either growth or lysine productivity.

DISCUSSION

The role of anaplerosis on lysine production, as this is defined by the formation of oxaloacetate, has attracted significant attention in earlier research. Two critical milestones were the development of an enzymatic assay for the *C. glutamicum* pyruvate carboxylase and the sequencing of the *pyc* gene (18, 32), which confirmed the existence and operation of this pathway. The availability of the *pyc* gene sequence also made it possible to investigate the effect of its overexpression on cell physiology, which is the subject of this paper.

The first interesting observation comes from the fact that the carbon source appears to have a major effect on the transcriptional and translational control of pyruvate carboxylase, with lactate being the best carbon source for achieving maximum expression levels. This was verified by Western blot and enzymatic activity data and appears to be in accordance with similar literature reports (31, 32). Such an effect has been observed in the past in other organisms such as *Pseudomonas citronellolis* and *Saccharomyces cerevisiae.* In *S. cerevisiae,* the expression of both isoenzymes (*pyc1* and *pyc2*) of pyruvate carboxylase is influenced by the growth phase and the type of carbon source.

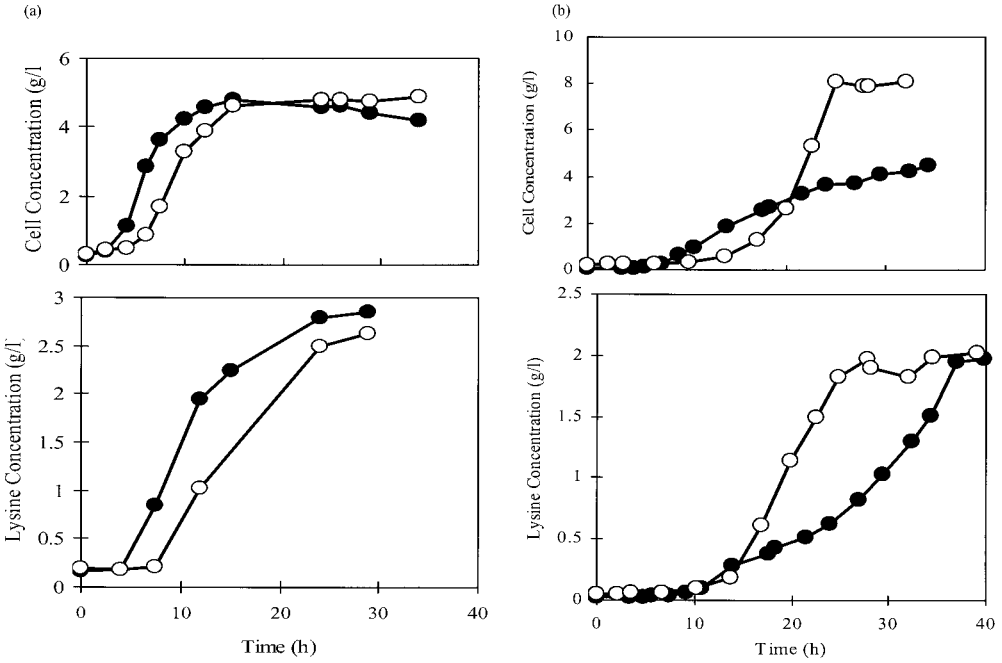

FIG. 5. Growth of and lysine production by parental and recombinant strain 21253 (a) and 21799 (b) cells grown on glucose. The upper and lower panels show cell growth and lysine production profiles, respectively. Solid and open symbols represent the parental and recombinant strains, respectively.

On glucose minimal medium, *pyc1* has a constant level of expression throughout the growth phase, in contrast to a high level of expression of *pyc2* only during the early growth phase. In ethanol minimal medium, the growth-related patterns of *pyc1* and *pyc2* expression were similar and showed a decline from early log phase to mid-log phase. The expression of *pyc1* plays an important anaplerotic role in maintaining fermentative growth and more notably in establishing gluconeogenic growth. On the other hand, *pyc2* expression seems to support growth on a glycolytic carbon source (3, 23).

For *P. citronellolis,* it has also been shown that the activity of pyruvate carboxylase is controlled by the carbon source (40). The activity of the enzyme is highest in cells grown in lactate or glucose and virtually absent in cells grown in malate or aspar-

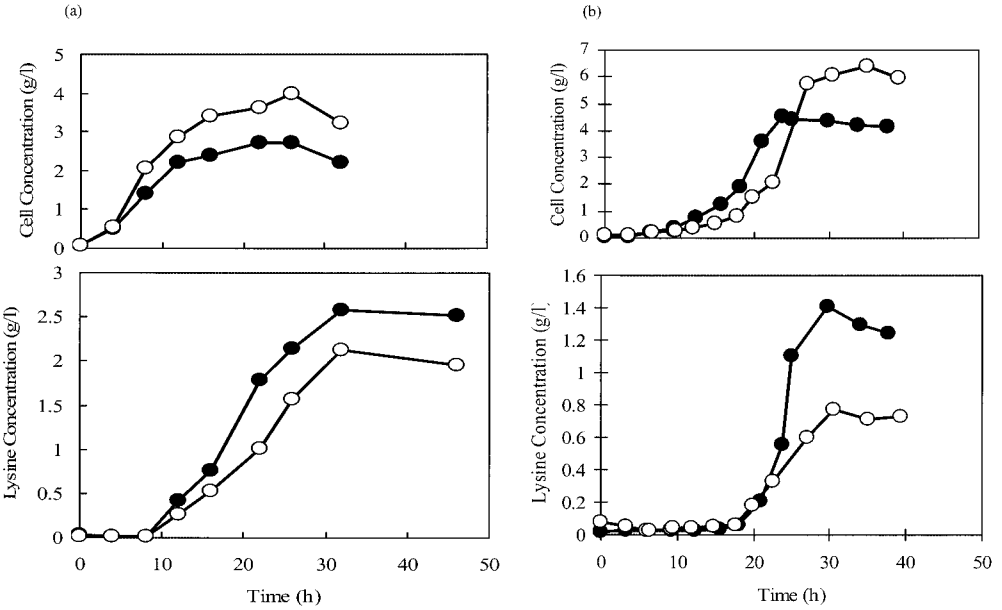

FIG. 6. Growth of and lysine production by parental and recombinant strain 21253 (a) and strain 21799 (b) cells grown on lactate. The upper and lower panels show cell growth and lysine production profiles, respectively. Solid and open symbols represent the parental and recombinant strains, respectively.

TABLE 4. Comparison of growth of and lysine production by *C. glutanicum* ATCC 21253 and ATCC 21799

Strain	Glucose			Lactate		
	μ^m (h^{-1})[a]	q_m (mg of lysine/g DCW/h)[b]	X_f (g/liter)[c]	μ_m (h^{-1})	q_m (mg of lysine/g DCW/h)	X_f (g/liter)
21253	0.35	46	4.2	0.34	63	2.5
21253(pKD7)	0.34	46	4.2	0.41	23	4.0
21799	0.18	32	4.2	0.16	53	4.1
21799(pKD7)	0.23	20	8.0	0.2	20	6.3

[a] Maximum specific growth rate.
[b] Maximum specific lysine production rate.
[c] Final cell concentration.

tate. This study also showed that coordinated regulation occurs at the level of synthesis of the two polypeptides, which make up pyruvate carboxylase in this strain, rather than at the stages of their assembly into protomers or the biotinylation of the apoenzyme (40). What is even more interesting is that this pyruvate carboxylase shows no control of its catalytic activity via effectors, such as, for example, acetyl-CoA, aspartate, or palmityl-CoA. In most varieties of pyruvate carboxylases examined so far, the enzyme appears to be constitutive, with regulation accomplished either through effector modulation of holoenzyme activity (pyruvate carboxylase from animal sources, yeast, or several species of bacteria) or through control of the biotinylation of the apoenzyme by biotin ligase (*Bacillus stearothermophilus*) (4, 5, 38). Finally, two other organisms where the carbon source controls the pyruvate carboxylase expression are *Azotobacter vinelandii* (37) and *Rhodobacter capsulatus* (25, 44).

Most interesting are the pyruvate carboxylase activity differences obtained on *pyc* overexpression in two different host strains and in two different carbon sources. When cells were grown in lactate and harvested in their late exponential phase, both recombinant strains [21253(pKD7) and 21799(pKD7)] showed higher pyruvate carboxylase activities than the parental strains. In glucose, however, enhanced pyruvate carboxylase activity was found in permeabilized cells of strain 21799(pKD7) but not in those of strain 21253(pKD7). In light of the *pyc* overexpression evidenced by Western blot analysis (Fig. 3), the lack of activity enhancement suggests a possible inhibitory effect on pyruvate carboxylase in host strain 21253. A possible explanation might be that aspartate, a common pyruvate carboxylase inhibitor, also inhibits pyruvate carboxylase in *C. glutamicum.* Strain 21253 contains chromosomal aspartate kinase that is inhibited by threonine and lysine. Since enzymatic activity measurements were performed in late exponential phase, when both lysine and threonine are present in the medium, the aspartate kinase activity is most probably still attenuated. This would result in intracellular aspartate accumulation and hence in a significant inhibition of pyruvate carboxylase activity, until threonine was depleted during stationary phase. The measurements of pyruvate carboxylase activity inhibition (Fig. 4) and intracellular aspartate accumulation (Table 3) are in accordance with this hypothesis for growth in glucose. On the other hand, when cells are grown in lactate, transcriptional activation (32) counterbalances the inhibitory effect of aspartate. No such effects appear in strain 21799 (pKD7), where aspartate kinase is deregulated and no apsartate accumulates intracellularly. Similar inhibitory effects by aspartate, as presented here, can also be found in other prokaryotic strains, including *Pseudomonas aeruginosa, Streptococcus faecalis, S. faecium, S. lactis, Micrococcus cerificans, Bacillus megatrium, B. subtilis, B. lichenformis,* and *B. stearothermophilus* (1, 13, 14, 20, 21, 33, 34).

The pyruvate carboxylase activity results in Table 2 are reflected in the phenotypes reported in Fig. 5 and 6. First, since there is hardly any difference in the pyruvate carboxylase activity between the parental and recombinant 21253 strains in glucose, the corresponding growth and lysine accumulation profiles are rather similar (Fig. 5a). The specific growth rate of the control strain was almost identical to that of the recombinant (0.35 and $0.34 h^{-1}$, respectively), and the same is true for the maximum specific lysine productivity (Table 4). Second, when lactate was used as the carbon source, enhanced growth and reduced specific lysine productivity were observed for both strains 21253(pKD7) and 21799(pKD7) relative to their controls (Fig. 6). Finally, recombinant strain 21799 grown in glucose (Fig. 5b) also showed enhanced growth and reduced specific lysine productivity in accordance with the activity results of Table 2.

The most surprising and counterintuitive result is arguably the lack of any effect of *pyc* overexpression on lysine production (Fig. 5a) or the downright reduction in specific lysine productivity (Fig. 5b and 6). This seems to be in conflict with prior reports according to which pyruvate carboxylase is a most critical step in lysine biosynthesis, accounting for as much as 90% of the anaplerotic carbon converted to amino acids (28). Instead of resulting in lysine overproduction, *pyc* overexpression reduced specific lysine productivity and increased both the biomass production and growth yields for both strains on lactate and for strain 21799 in glucose. This result suggests a kinetic limitation of growth by pyruvate carboxylase that was released by the *pyc* overexpression. Put differently, an increase of the pyruvate carboxylase activity alone, without a commensurate increase in the activities of aspartate kinase and other enzymes downstream in the lysine pathway, increases the supply of tricarboxylic acid TCA cycle metabolites that are also precursors of biomass synthesis. This results in greater biomass growth, further draining other biomass precursors from the aspartate amino acid family and thus reducing lysine formation. Under these conditions, the net result of *pyc* overexpression is a metabolic flux redistribution at the key branch- points of phosphoenolpyruvate, pyruvate, and oxaloacetate to favor biomass production relative to product (lysine) synthesis.

Obviously, such a metabolic flux redistribution can be prevented by a simultaneous enhancement of other rate-controlling enzymes in the lysine pathway so that an overall activity balance is maintained through the entire product pathway following *pyc* overexpression. The fact that this balance is disturbed on *pyc* overexpression suggests that other potential bottlenecks emerge in the linear pathway leading from aspartate to lysine after the activity of pyruvate carboxylase has been significantly enhanced. Such possible bottlenecks identified in the past include aspartate kinase and diaminopimelate synthase. Combining the overexpression of pyruvate carboxylase with these enzymes is an obvious next target in the amplifica-

tion of the lysine production pathway by metabolic engineering.

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